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AWARD NUMBER DAMD17-97-1-7310

TITLE: Breast Tumor Detection Utilizing Novel in situ
Amplification Systems

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REPORT DATE: August 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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20001107 053

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE August 1999	3. REPORT TYPE AND DATES COVERED Final (7 Jul 97 - 7 Jul 99)		
4. TITLE AND SUBTITLE Breast Tumor Detection Utilizing Novel in situ Amplification Systems		5. FUNDING NUMBERS DAMD17-97-1-7310		
6. AUTHOR(S) Cassandra L. Smith, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Boston University Boston, Massachusetts 02215		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)				
<p>Although existing radiographic, ultrasound and MRI modalities for the the detection of breast cancer are useful, they are less than ideal. Nuclear medicine approaches using radiolabeled anti-breast tumor antibodies and other radiolabeled agents have met with similar mixed results. Accordingly there is a need for improved approaches for the detection and treatment of breast tumors. This work explored several novel approaches using DNA for specific targeting and signal amplification. The work completed during this period will serve as the foundation for future work in this area.</p>				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 17	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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CSnell
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5. INTRODUCTION

Considerable interest has been generated in the use of nucleic acids for therapeutic use (1-3). Exciting examples of anti-sense applications include selective inhibition of viral RNA replication or gene expression (4-6). Other in vivo applications have included oligonucleotide-antibody conjugates for tumor diagnosis and therapy (7).

This interest promoted the investigation in model systems of the pharmacokinetics, biodistributions and stability of oligonucleotides in vivo ranging in size from 12 bases (8) to as large as 38 bases (9) with radiolabels, such as ^{125}I (7,10,11), ^{32}P (9,12), ^{35}S (12, 13, 14) and ^{111}In (15, 16). In these and other in vivo applications the nucleotide backbone was modified to prevent nuclease degradation, typically with methylphosphonates (17-20) or phosphorothioates (21-24), although terminal modification alone was shown to provide stability (9, 13, 20, 25).

Amplification techniques for nucleic acids are under active investigation for in vitro applications. These include PCR (26), the "christmas tree" approach (27), and branching nucleic acid dendrimers (28-34). However, using nucleic acids for amplification in vivo has not previously been considered.

The overall goal of this project is to form at the site of a tumor a large aggregate. The complex is formed, de novo, at that site of tumor from small molecules which self-aggregate. The formation of the aggregate means that the original tumor target signal is amplified. Then, the aggregate at the site of the tumor is used as the target of a small molecule carrying a low molecular weight label such as an imageable radionuclide. This proposal describes the assembly in situ of an aggregate possessing what could be an almost limitless amount of radioactivity while at the same time the method seeks to minimize radioactivity in normal tissue. Thus, the tumor tissue radioactivity ratios should be exceptionally high for both diagnostic and therapeutic applications.

This approach is based upon several well characterized high affinity

systems (double stranded DNA, streptavidin-biotin, and leucine zipper dimerization) that are being combined in a unique fashion. Nucleic acids and their derivatives were chosen because (1) the affinity and specificity between two complementary DNA sequences in natural settings is quite efficient and tight, (2) short DNA molecules should be non-toxic and (3) DNA molecules can be radiolabeled relatively easily via a number of strategies. A streptavidin-biotin system was chosen because of (1) the high stability of this protein in vitro and in vivo and its low immunogenicity, (2) the existence and ongoing construction, of a variety of potentially useful recombinant streptavidins, (33), the ease of biotinylation of a wide variety of molecules. The dimerizing leucine zipper recombinant constructs was chosen because of (1) the specificity and stability of the dimer and the fact that the zipper can be formed with a short oligopeptide. The three novel amplification strategies to be tested here are (1) a protein-nucleic acid aggregate consisting of a pair of streptavidin conjugated with complementary single stranded DNAs (or PNAs), (b) a pure protein aggregate consisting of pairs of streptavidin fused to the ends of the leucine zipper. One zipper is fused to the N-terminus of streptavidin and the other is fused to the C-terminus. It is also anticipated that the nucleic acid amplification schemes described herein will have applications which extend far beyond this proposal.

The formation of aggregates at tumor sites can be broken down into three stages (1) initialization, and (2) amplification and (3) termination. Although the primary focus on this research was on the amplification stage, exciting results on other experiments focused on the initializing step lead up to concentrate our efforts in that area.

The initializing or targeting of reagents to tumor cells critical to this and other efforts to treat cancer. In fact, a major problem in medicine is delivering a therapeutic agent to a target organ. Currently, most medication is not targeted but rather distributed throughout the body. This means that therapeutic levels are achieved after excess amounts of medication is given to a patient. Drug doses are chosen that provide an effective level to the target cells and minimize damage to bystander tissues. A recent elegant solution being explored by some researchers is to place medication on magnetic beads and then target the appropriate organ using an external magnet. This may be appropriate for large organs

like the heart, etc. However, this approach is not likely to work with solid tumors because of multiple micrometastases at secondary sites being a primary factor morbidity.

Antibody targeting of tumor cells does not work well. In general, tumor specific antibodies have been difficult or impossible to develop. The problem initialization was in fact highlighted as the major criticism of the original proposal. Antibodies tend to get stuck in the kidneys and have problems penetrating solid tumors. Hence, experiments, supported by other funds were used to explore the use of nucleic acids for tumor targeting. The goal of those experiments was to isolate a DNA mimics of antibodies, called aptamers (meaning in Greek, "to fit") specific for tumor cells. The advantage of aptamers is their small size and the ability to select specific, high affinity aptamers from large libraries of random nucleic acid sequences. Nucleic acids are non-immunogenic. When other funds, supporting that work ran out, the aptamer project was continued with funds from this project. Progress on initialization step (aptamer project) and amplification step will be discussed below.

6. BODY

Materials: Oligonucleotides used in this work were purchased from Operon Technologies (Alameda, Ca). Where needed the nucleic acid will be fluorescently labeled for direct imaging of aggregates with fluorescent digital microscope or end-labeled by us with ^{32}P using conventional DNA kinasing methods. Biotinylated oligonucleotides were purchased as needed. Streptavidin coated microbeads were purchased from Dynal Inc. (Oslo, Norway). Tumor cell lines were purchased from the American Type Culture Collection (ATCC). DNA sequence analysis was done using GCG software provided by the BMERC facility of Boston University and by freeware MEME and MAST. DNA sequencing kits were purchased from Pharmacia. Carcinogenic embryonic antigen (CEA), concanavalin A and alpha-mannose was purchased from Sigma. Chemically reactive biotin was purchased from Aldrich. DNA cloning and sequencing was done using commercial kits from Clontech and Pharmacia, Inc. The automatic fluorescent ALF-express sequencer was used for sequence determinations.

Methods: (A) Aggregate Formation: Several very simple systems were

set up to explore aggregate formation in solution and on surfaces. A general schematic of the approach for in vivo targeting is illustrated in Figure 1. A first step (initializing step) would involve the administration of a tumor-specific molecule tagged with a linker to the aggregation system. This could be an antibody directly conjugated with single stranded DNA molecules or an anti-tumor aptamer with a single stranded tail. This is followed by the administration of a streptavidin conjugate (or pure DNA species in the form of a Holliday structure with four single stranded ends). Potentially there are four binding sites on tetrameric streptavidin for biotinylated oligonucleotides to bind. The single stranded regions should be at least 20 to 24 bases in length and one of the single stranded oligonucleotides/streptavidin molecule complementary to the single stranded species on the initializer. The third step in the procedure is the addition of a second streptavidin species with oligonucleotides complementary to the remaining single stranded ends. Alternating injections of complementary oligonucleotides should produce a large aggregate at the site of a tumor. The final step would be the addition of an radiolabeled single stranded DNA, complementary to most recently injected oligonucleotide.

Potentially, each cycle of addition of complementary DNAs strands for tetra-valent reagents, will result in an up to three-fold increase in the number of strands added to the aggregate. This amplification is potentially greater than that of PCR which makes it an attractive candidate for in vitro applications as well. Using these methods it should be possible to construct an aggregate at each antigenic site which contains an exponential increase in the amount of radiolabeled oligonucleotides achievable by direct labeling experiments. Moreover, the radioactivity should be concentrated at the tumor due to the quick clearance of the low molecular weight radiolabeled strands from circulation and normal tissues.

(B) Anti-CEA Aptamers: CEA was chosen as a first aptamer target for tumor targeting because of the enormous amount of work over the last 40 years using anti-CEA antibodies. Although anti-CEA antibodies are effective targeting reagents, the large number of anti-CEA antibody experiments can serve as standards for establishing the behavior of anti-CEA aptamers. Potential anti-CEA aptamers were selected from random DNA oligonucleotide libraries with a complexity of $\sim 10^{15}$ using methods

developed by us (Smith et al., unpublished results). A DNA library for aptamers was prepared from a 100-base length single stranded DNA. The DNA oligonucleotides has a 64-base variable sequence flanked by two 18-base constant sequences. The constant sequences are used as primer-binding sites for PCR amplification. These experiments were begun using a conventional aptamer selection approach. Conventional aptamer selection methods incubate an immobilized target molecules, in this case CEA, with an oligonucleotide library of random sequences. This set up allows unbound oligonucleotides to be removed efficiently. The conventional aptamer selection protocol was used without success. It then became apparent that the failure was because the selection protocol was diffusion limited by the low concentration of CEA and further by its immobilization. This meant that CEA would not interrogate a large portion of the library unless extensive (weeks) incubation times were used. Hence, the procedure was modified so that CEA was incubated with the oligonucleotide library in solution. After sufficient time, the target plus bound oligonucleotides were immobilized and the unbound oligonucleotide removed. Using the modified selection protocol, a library of potential anti-CEA aptamers was created. The success of the selection protocol is likely because (1) about 20% of the selected library bound to CEA after 7 rounds of selection when <1 % bound initially and (2) specific restriction fragments could be detected in the oligonucleotide pool after 4 rounds of selection, while none were detected in the first three selection rounds. In this work, the library from after the seventh round of selection was cloned and sequenced using conventional methods. The sequences were analyzed using several multiple alignment program to identify shared motifs.

Results: (A) **Aggregate Formation:** Two model systems were set up to study layer formation in vitro. In addition, studies on a simple tumor model system was also begun. One in vitro system followed aggregate formation in solution and the other followed aggregate formation on a surface. The solution system used streptavidin coated beads. The beads were divided into two batches. Biotinylated complementary DNAs were used to link the beads together (Figure 2). On average, the aggregations were composed of 4 beads in diameter. In the surface aggregation system, fluorescently labeled streptavidin was used to follow the formation of the aggregation. One can envision such a surface as the outside layer of a

tumor. The results of such an experiment are shown in Figure 3. The quantitative data shown that a 50-mer linker allowed four layers to be build up. AT this time it is not clear why the aggregate size could not be expented beyond four layers but there are many vaiables to be tested. However, it may be that the geometry of the biotinylated DNA stranded emerging fromt he bioting binding sites of streptavidin sterically hinder the construction of more layers. This means that the formation of aggregates might best be done using pure DNA molecules.

The model system developed with tumor cells involved the immobilization of single stranded DNA on the surface of a tumor cell. This oligonucleotide served as the initializing target to study the formation of an aggregates on tumor cells (Figure 4). The protocol involved developing a protocol for biotinylating the surface of a whole tumor cells without affecting viability using standard chemical methods. Then, adding streptavidin with biotinylated oligonucleodies. After this aggregate formation would proceed as outlined above. The experimental results demonstracted that tumor targeting in this model sytem could be accomplished with DNA and that the DNA was stable on the cell surface for at least 24-hours.

(B) Anti-CEA aptamers: About 100 individual oligonucleotides were cloned and sequenced using conventional methods after 10 rounds of selection when about 20% of the library bound to the target (Table 1). After four rounds of selection, specific restriction fragment cleavage sitescould be detected in the the selected pools (Figure 5) indicating that the complexity of the pool was greatly reduced. Several multiple alignment programs were used to compare the sequences and develop a minimum unique sequence group (Table 2). Several software programs including PILEUP in GCG and MEME and MAST were used to search for conserved sequence motifs in the minimal set. Three motifs were identified. The affinity of these sequences for CEA are now being measured.

7. KEY RESEARCH ACCOMPLISHMENTS

The goal of this research is to use DNA as therapeutics for cancer tumor cell treatment. The research program explored a number of aspects of this approach and has produced a foundation on which additional research can be done. Here, the initialization and amplification steps were explored. The most significant accomplishment was the isolation of potential anti-CEA aptamers. The other accomplishments include the set up of model systems using tumor cells and beads.

8. REPORTABLE OUTCOMES

Grant Applications:

Cassandra L. Smith (PI), Tumor Targeting with DNA Aptamers, DOE, 1998
- not funded

Cassandra L. Smith (PI), Anti-Tumor Targeting, Boston University Provost Innovation Fund, 1999 - funded \$25,000

Gregg Surdi, Pre-doctoral Fellowship Application. Targeting Breast Cancer with Anti-CEA, DOA Breast Cancer Program, 1999 - pending

Presentations:

Cassandra L. Smith, 1999. Physics Colloquium at Boston University, "Macromolecular DNA Structures"

Patent Application:

Disclosures about this technology have been given to the Boston University Patent Office who is decided on whether to file patents.

Publications:

It is anticipated that there will be at the minimum one publication from

this work once the work is completed, although it is more likely to be multiple publications from this work.

9. CONCLUSIONS

The funds provided by the Department of Army enticed the principle investigator to begin thinking about breast cancer disease and to develop a completely new line of research based on DNA therapeutics. As with truly new research directions, progress has been slower than predicted, but promising enough so that this work will continue even in the presence of very limited funds.

The principle investigator believes that the demonstration of the specificity of the aptamers is key for demonstrating the feasibility of the using DNA therapeutics for breast cancer disease treatment. Hence, the current focus of our research is on confirming the specificity of the aptamers that have been isolated to provide the initializing step

The amplification (aggregate) step needs to be further developed theoretically and in vitro as there is little published research in the area of homogenous and heterogenous molecular structures in vitro. Some of the most promising work has been reported by Seeman, 1995, 1998. It is quite clear that nucleic acids are versatile tools for building molecular structures for a variety of purpose because they allow fast prototyping and virtually any sequence can be made rapidly and easily. Hence, it is anticipated that this work will continue once more funds are available.

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Tables 1 Binding of oligonucleotide libraries to CEA after each round of selection. The percent bound was determined by measuring the amount of ³²P-labeled oligonucleotide immobilized with CEA.

Selection Rounds	CEA $\mu\text{g}(\mu\text{M})$	DNA $\mu\text{g}(\mu\text{M})$	Unbound (ml)	Bound (ml)	% Bound
1	200 (~2)	76(~4)	5.7	7.2	5.99
2	200	27	6.5	6.4	0.91
3	200	6.4	7.0	7.0	0.42
4	200	10	7.3	7.2	0.61
5	200	9	17	7.5	2.93
6	200	6.7	19.5	7.5	18.9
7	-	3.3	-	-	-
8	-	3.3	-	-	-
9	200	2.9	20	7.0	9.9
10	200	3.2	28	7	21.1

Table 2. Potential anti-CEA aptamers. After the tenth round of selection, oligonucleotides were clones and sequenced. A minimum unique set of aptamers were aligned and three recurring motif were identified.

```

clone45 ----- --GGTGGGGG GATTCCCTCG TTTATTACG TYTCTGAYAT GGTTCCGCTC ACGCTCCCC T
clone48 -----GGATG GTAGTCGGGG TATCCTTTTA AGGAGTTAGT CAACGAGCGT TAGTATTTTA ATGTTCCC~
clone60 ---AGACGAG GBMAGTGGGG sAGTACCY.V CTV.GVAGTT TAADGSSB.K BCBTAT.ATT CCACGGBTGA T
clone66 ---AGACGA. GGAAGTGGGG GAGTACCC.G CTG.GGAGTT CAAAGCAT.T TCTTAT.ATT CCACGGCTGA T
clone52 ---AGACGA. GSAAGTGGGG GAGTACCC.G CTG.GGAGTT CAAAGGGBGB SCBTAT.ATT CCTCGCCCC~
clone72 ---AGACGA. GGAAGTSGGG GAGTACCC.G CTG.GGAGTT CAAAGCAT.T TCTTAT.ATT SCTCGCCCC~
clone16 ---GGVAGAV GG.TGAGGSG GCGTACCC.G CCGTGGAGGA TATCGGTATT AGACATCAGT TCTTTCCG~
clone22 ---GGGGGAG GGGGCGACGC GATACCC.G CTTAGAT.TA TACGTTTGGT ATTCAATTCT GCCGCCCTT~
clone39 ---GGGGGAAG GGGGCGACGC GATACCCCG CTTAGAT.TA TACGTTTGGT ATTCAATTCT GCCGCCCTT~
clone75 ---GGGGTAG GGGSCGACGC GATACCCS.G CBBVGVT.TA TACGTTTGGT ATTCAATTCT GCCGCCCTT~
clone23 ---AGGGGAAG GGGCTGAAAG GATACCCCTG TTGATCTGTG TAACACCAT? AGTTATATCT ACTTCC----
clone74 ---GGGGTAG GGGGCGAAGC GATACCCCTG NTGATCTGTG TAACVCSVSS VGBTATATCT ACTTCC----
clone26 ---GGGGTAG GGGGCGAAGC GATA.CCCTA ATCAGCGTCC TA..GCGGCT TCCGGTTTCG TCTTTTCG~
clone11 ---GGCGAG GGGAAAGGAG GAGTCCTAAG GGGTACTGTA GGGAGYTRGT TTTTAAACCT CCTTTT----
clone78 ---GGGGGG GTAGGGGGCC TAGCCCTCTT CATTAATK.. GGRAGCCGCG TTTTCTCGA G-----
clone41 ---ACCATGAGGG GGGGCGTAAC CCTACAAGCA TACGCATGGC TATTTCTTCA GCGTCTCGTA~
clone57 -GCAGGAGTG TGAAGGAATT GATGG.GGgC GGGGGGTGAA GCGACACSCC TTTCAACTCT TCGTCG----
clone62 ---GGGAG GGTGGG.ACG GTTGC.GGTG GTTGGGGTAA GGCCCAACCSC CTTTAGC.CT TCGVTTTCCC T
clone9 AGAAGGGGGG ACCAGGGCTA GTGACTGGgC ATGAAGTGAA GGACGGTACA TTCCAATTGG CTGT-----
clone51 ---ATGATA GAGG.TGGGT GCGCTCCTTG A.GGTGGGGA GGGGGACGAG TGTGACACCC CGGTTG----
clone71 -ATCGGGATT GTSGTTCCGA GATGCCATTG ATAGTGAYGA TTTYNNVCRG .GTGAACCCG CTGTGG----
clone76 ---GGNGGC TTGGTCCGGT GAATTCCTGA AGAGTTTAT TCTAGACTCG CTTGTTTCTT TGCCAG----

```

Motif 1
AGGGGGTGAAGGGATACCC
G AATC GGACAG T
A C

Motif 2
TATTTTTTTTCG
GACACCA T
A

Motif 3
CTGCTGATCTGTGTAA
C TGTTAT C CG G
G

Figure 1. Schematic of aggregates formed using Streptavidin-Streptavidin-biotinylated linkers. Circles represent tetrameric streptavidin. Bis-biotinylated double stranded DNA serves as linker between streptavidin molecules.

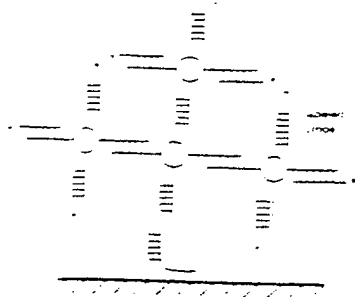
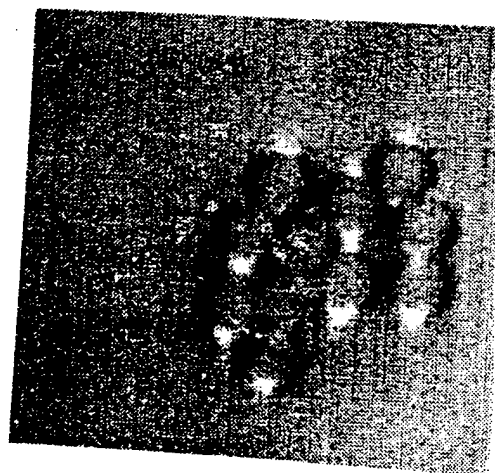


Figure 2. Formation of Streptavidin coated microbead-biotinylated DNA aggregates in solution. (A) aggregates of streptavidin coated microbeads through a bis-biotinylated double stranded DNA. (B) Beads with non-complementary DNAs do not aggregate.



B

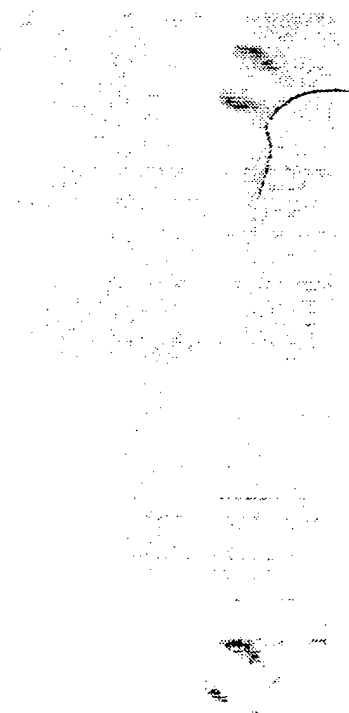


Figure 3. Formation of Streptavidin-biotinylated DNA aggregates on a silicon surface (see text for details)

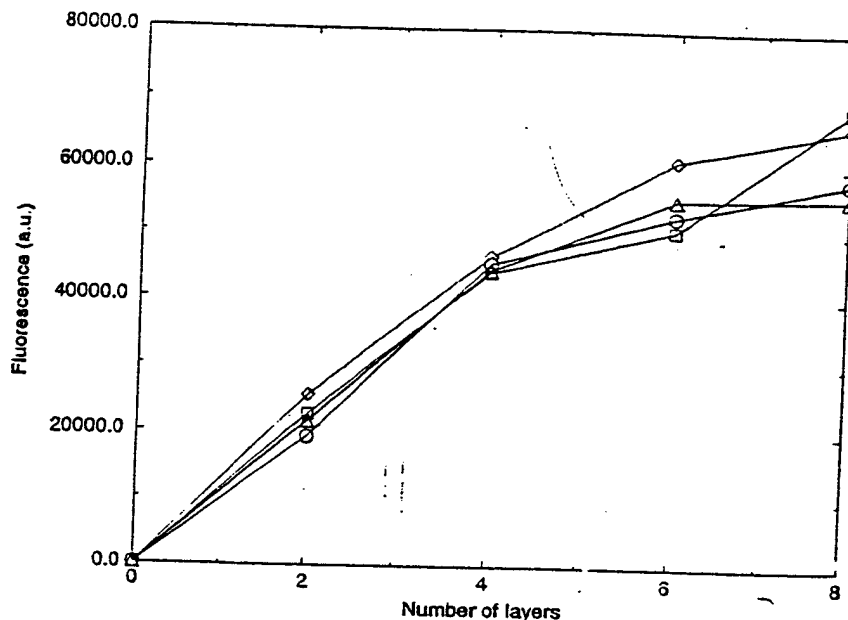


Figure 4. Target single stranded DNA immobilized on the surface of tumor cells. Breast cancer tumor cells grown in tissue culture were treated with chemical methods with doses that did not affect viability. The cells were incubated with streptavidin followed by incubation with biotinylated DNA then followed by incubation with complementary (A) or non-complementary (B) ^{32}P -labeled DNA.

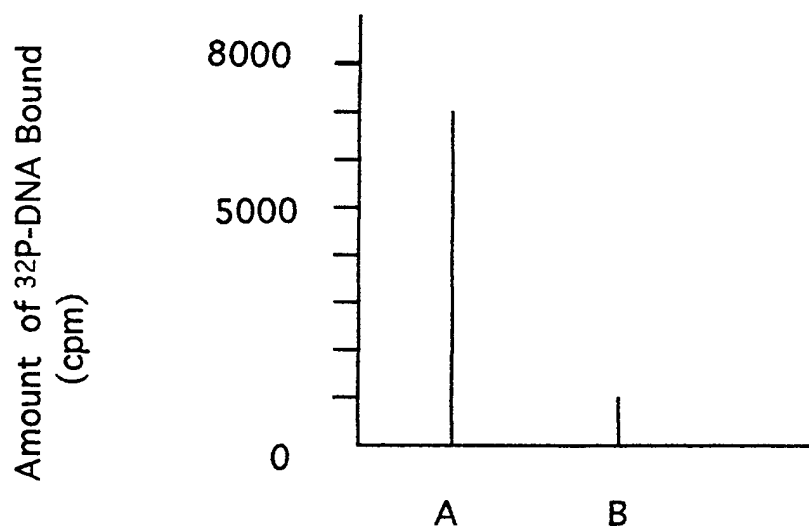


Figure 5. Oligonucleotide library Complexity. After each selection cycles a fraction of the DNA was re-amplified by PCR with one ^{32}P -labeled primer. The 100 base PCR products were gel purified using a Qiagen kit and digested with three restriction endonucleases (Sau3A I, Hinf I and Acl I) and fractionated by size on a denaturing 8% polyacrylamide gel containing 7 M urea. The occurrence of specific bands is indicative of low complexity samples.

